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Studies on the Concentration of Arsenic, Selenium, Copper, Zinc and Iron in the Blood of Blackfoot Disease Patients in Different Clinical Stages

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Summary: Flame atomic absorption spectrophotometric methods were developed for the determination of zinc, copper, arsenic, iron and selenium in blood samples. Data from blackfoot disease patients in five clinical stages were compared with those from healthy controls. Copper concentrations were the same for all clinical stages. Arsenic increased in the initial three stages but decreased thereafter, although arsenic was previously considered to be the major causative agent of the disease. The decrease of arsenic in the later stages was attributed to the antagonistic effect of selenium, and the decrease of iron during the progress of the disease is thought to be due to the antagonistic effect of arsenic in the initial stages and the loose of haemoglobin in the later stages.

Introduction

An endemic peripheral vascular disease called blackfoot disease is suffered by a large number of inhabitants on the southwest coast of Taiwan (1–6). The disease has an insidious onset with numbness or coldness as the initial symptom. It progresses with the development of localized ulceration and subsequent gangrenous changes, giving the characteristic black coloration of dry gangrene. Most patients are permanently maimed as a result of spontaneous or artificial amputation of a portion of the affected extremity (7).

Blackfoot disease is thought to be related to the presence in artesian drinking water of high concentrations of arsenic, silicate, copper, nickel and certain fluorescent compounds, with arsenic as the primary suspect (8–18).

Patients with advanced clinical symptoms should therefore have a high concentration of blood arsenic, but our preliminary results showed a decrease of arsenic in the advanced stages. In order to assess this situation, we determined arsenic, iron, selenium, zinc and copper in blood samples from blackfoot disease patients in different clinical stages. The results indicate a probable antagonistic effect between arsenic and selenium.

Materials and Methods

Blood samples

One hundred and thirteen blood samples of blackfoot disease patients at five clinical stages according to the criteria set by Drs. *U. C. Huang* and *D. T. Lin* (tab. 1), and 49 blood samples of persons with no known history of exposure to arsenic were used. As shown in table 2, there was an even distribution of age and sex between the two groups. Samples were frozen below −20 °C until used.

Chemicals and biological standards

Suprapur grade reagents of E. Merck and high purity water (18 MΩ) were used. Stock solutions containing 1000 mg/l or mg/kg of iron, zinc, copper, selenium and arsenic and working

Tab. 1. The different stages of blackfoot disease as classified in Chi-Yi hospital, Taiwan

Stage	Symptom
Zero stage	Assumed to be present in residents of endemic area without evidence of disease.
First stage	Coldness, numbness and pain.
Second stage	Evidence of slight ulceration and slight gangrenous changes.
Third stage	Evidence of definite ulceration and definite gangrenous changes.
Fourth stage	Evidence of gangrenous changes of the affected extremity. Spontaneous or artifical amputation of foot.

Tab. 2. The distribution of specimens

		Different stages of blackfoot disease				
	Controls	Zero stage	1st stage	2nd stage	3rd stage	4th stage
Male	26	14	20	13	9	21
Female	23	5	14	5	2	10

Note: The ages of the patients and healthy control persons were about 65 ± 10 years.

standard solutions were prepared from Merck Titrisol standards by diluting with the high purity water. Containers made of quartz, Teflon, or polypropylene were used throughout. They were immersed in 8 mol/l HNO_3 overnight and washed with several changes of distilled water. Biological standards were NIES human hair No. 5, and NBS serum 1598.

Analytical methods

Atomic absorption spectrophotometer model Z-8000 and its accessory Hydride Formation System HFS-2 from Hitachi, Japan were used for determining trace metals. Prolab Maxdigester 350 (France) was used for sample digestion.

To 10 ml of a whole blood in a digestion flask were added 20 ml of conc. HNO_3. The power output of the digester was maintained at 15% for 15 min and at 25% for 10 min. After adding 7 ml each of conc. H_2SO_4 and $HClO_4$, the digestion was continued at 30% power for 10 min and at 40% for 35 min. Finally 10 ml of water was added and the power kept at 40% until a colourless solution was obtained (taking about 8 min). The digest was diluted to 50 ml with the high purity water. Aliquots were taken for the ordinary (iron, zinc and copper) and the hydride (arsenic and selenium) modes of atomic absorption spectrophotometer.

Results and Discussion

Data accuracy and analyte recovery

To check the data quality, human serum and hair standards were analysed. As shown in table 3a, the best accuracy, expressed as the % coefficient of var-

Tab. 3a. Recoveries for the analysis of NBS serum by HFAA and FAA methods (n = 6)

NBS 1598 Serum	FAA** Fe (mg/l)	FAA Cu (mg/l)	FAA Zn (mg/l)	HFAA* Se (mg/l)
Analysed	214 ± 8	0.71 ± 0.06	0.86 ± 0.04	0.03 ± 0.004
CV (%)	3.7	8.4	4.6	11.1
Certified values	225 ± 9	0.72 ± 0.06	0.89 ± 0.06	0.042 ± 0.004
CV (%)	4.0	8.3	6.7	9.5
Recovery (%)	95	98	96	84

* HFAA: Hydride flame atomic absorption.
** FAA: Flame atomic absorption.

Tab. 3b. Recoveries of the analysis of NIES human hair by HFAA and FAA methods (n = 6)

NIES No. 5 Human hair***	FAA** Fe (mg/kg)	FAA Cu (mg/kg)	FAA* Zn (mg/kg)	HFAA Se (mg/kg)
Analysed	2.55 ± 0.15	15.5 ± 0.7	165 ± 9.0	1.15 ± 0.25
CV (%)	6.0	4.5	5.4	21.7
Certified value	2.55 ± 0.10	16.3 ± 1.2	169 ± 10	1.4****
CV (%)	3.9	7.3	5.9	—
Recovery (%)	100	95	97	83

* HFAA: Hydride flame atomic absorption.
** FAA: Flame atomic absorption.
*** N.I.E.S. National Institute for Environmental Studies (Japan-Environment Agency).
**** N.I.E.S. No. 5 Human hair reference value.

iation (CV%) and recovery of analytes from the serum standard, were less than 8.4% and 98% for copper, less than 4.6% and 96% for zinc, and less than 3.7% and 95% for iron, respectively. Selenium, however, had a larger CV% of 11.1 and a poor recovery of 84%, owing to its volatility. The results in table 3b indicated that the best accuracy (CV%) and recovery of analyses of the human hair standard were less than 6.0% and 100% for iron, less than 5.4% and 97% for zinc, and less than 4.5% and 95% for copper, respectively. Again, selenium had a high CV (21.7%) and a poor recovery (83%). The recoveries of arsenic added to whole blood of normal persons and blackfoot disease patients in the first clinical stage are compared in table 4. Patient samples showed a higher CV (14.1%) and a higher recovery (86.5%) than samples from controls (13.5% and 84.7%, respectively).

Recovery and standard curves

Standard and recovery curves of iron, zinc, copper, selenium and arsenic were obtained by analysing normal blood samples. To 1 ml of the sample were added the following:

0.4, 1, 2, 4, and 10 μg of iron;
0.2, 0.4, 1 and 2 μg of zinc and copper;
2, 4, 10 and 20 ng of selenium;
2, 4, 10 and 20 ng of arsenic.

The results are shown in figures 1 and 2. Good linear relationships were obtained for these concentration ranges. Good recoveries of 97, 95 and 91% were obtained for iron, zinc and copper, respectively. Poorer recoveries of 81 and 87% were obtained for arsenic and selenium, respectively, owing to their volatility.

Tab. 4. Recoveries of arsenic added to whole blood of normal persons and blackfoot disease patients in the first stage of the disease (n = 6)

Whole blood from	No. Specimens	As added (μg/l)	As recovered (μg/l)	CV (%)	Recovery (%)
Normal persons	12	40	33.9 ± 4.6	13.5	84.7
Blackfoot disease patients	2	40	34.6 ± 4.9	14.1	86.5

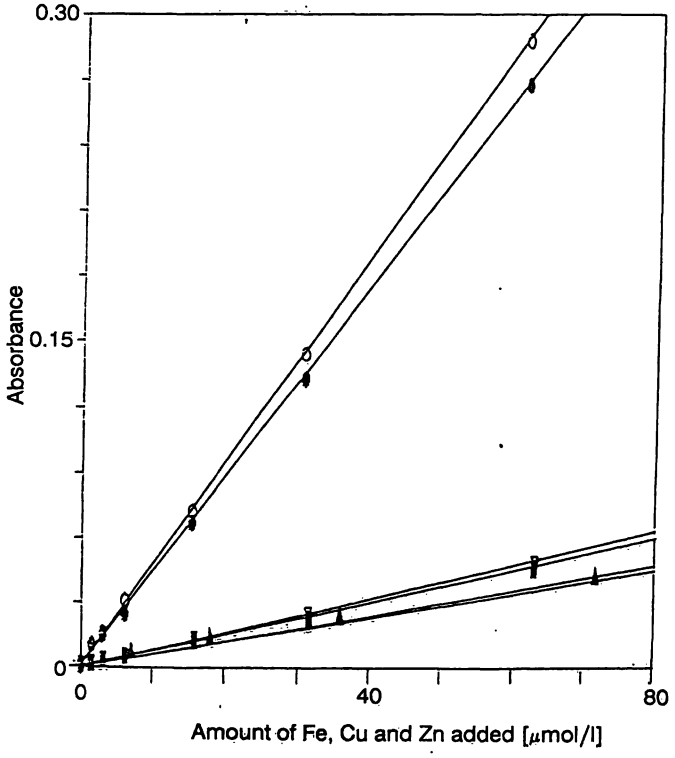

Fig. 1. Assay curves for iron, copper and zinc.
△ Fe in purified water ▲ Fe in normal person blood
□ Cu in purified water ■ Cu in normal person blood
○ Zn in purified water ● Zn in normal person blood

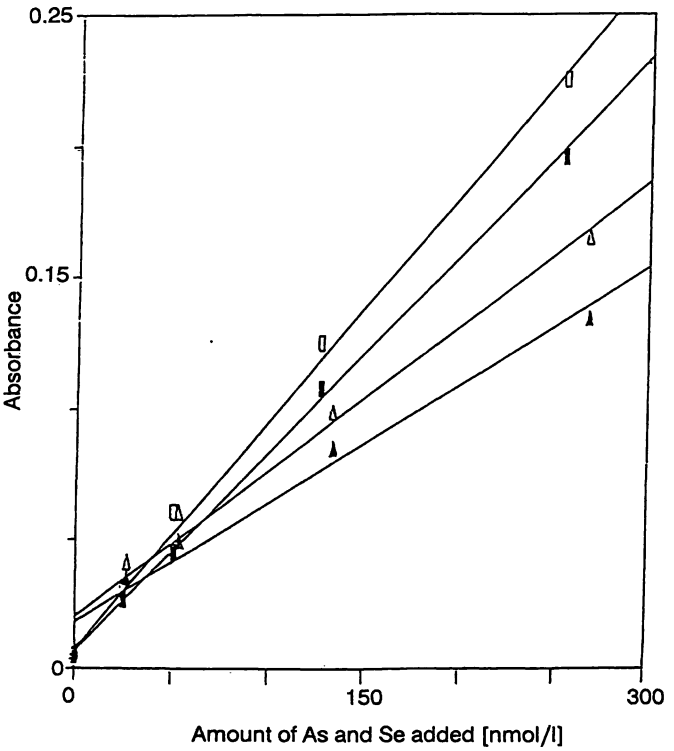

Fig. 2. Standard curves for the assay of arsenic and selenium.
△ As in purified water ▲ As in normal person blood
□ Se in purified water ■ Se in normal person blood

Tab. 5. Comparisons of blood arsenic, selenium, copper, zinc and iron concentrations of blackfoot disease patients and normal persons

	Normal persons n = 49	Patients n = 113	Deviation between normal person and blackfoot disease patients (%)
Arsenic (nmol/l)	89.4 ± 45.3	133.4 ± 72.0	+49
CV (%)	50	54	
Selenium (μmol/l)	0.79 ± 0.28	0.59 ± 0.30	−25
CV (%)	31	51	
Copper (μmol/l)	9.44 ± 4.09	9.11 ± 3.99	+ 5
CV (%)	43	39	
Zinc (μmol/l)	64.3 ± 21.4	62.5 ± 19.5	− 2.8
CV (%)	33	31	
Iron (μmol/l)	9.88 ± 1.66	11.5 ± 3.24	+17
CV (%)	16	28	

Analyses of blood samples

Table 5 shows the analytical results of blood samples obtained from the normal and diseased persons without discrimination of clinical stages. It shows that the blood zinc and copper concentrations of patients do not differ from those of normal individuals; they fall within the 5% deviation (zinc, 64.3 ± 21.4 and 62.5 ± 19.5 μmol/l; copper, 9.44 ± 4.09 and 9.11 ± 3.99 μmol/l), but arsenic, selenium and iron showed significant differences, with deviations of 49, 25 and 17%, respectively (arsenic, 89.4 ± 45.3 and 133.4 ± 72.0 nmol/l; selenium, 0.79 ± 0.28 and 0.59 ± 0.30 μmol/l; iron, 9.88 ± 1.66 and 11.5 ± 3.24 μmol/l). Patient blood samples contained lower selenium concentrations, but higher arsenic and iron concentrations than those of controls. Blood arsenic concentrations of patients in different clinical stages and living in higher endemic areas were compared with those living in lower endemic areas (tab. 6).

Tab. 6. Comparisons of blood arsenic concentrations of blackfoot disease patients from higher and lower endemic areas

	Higher endemic area patients	Lower endemic area patients	Deviation between the higher and lower endemic patients (%)
Arsenic (μg/l)	10.5 ± 5.6	7.7 ± 4.3	26
CV (%)	53	55	

Samples from patients in higher endemic areas had a significantly higher arsenic value of 10.5 ± 5.6 μg/l compared with 7.7 ± 4.3 μg/l in the lower endemic areas, with a deviation of 26%. A positive correlation therefore exists between the blood arsenic concentration and the onset of blackfoot disease.

Antagonism of arsenic by selenium

Figure 3 shows the analytical results of blood specimens obtained from patients in different clinical stages. It shows that the deviation of blood copper, zinc and iron for either diseased or normal persons were always within ± 5%. But arsenic and selenium had a larger deviation of more than ± 10%. The copper concentrations for either patients or normal persons have an average value of 9.67 ± 0.94 μmol/l.

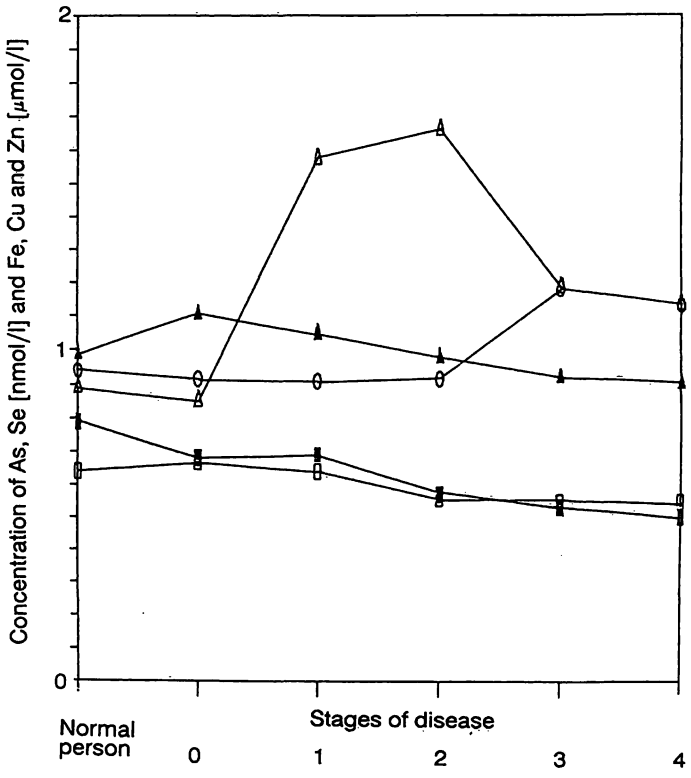

Fig. 3. Blood arsenic, selenium, iron, copper and zinc concentrations in different stages of blackfoot disease. Each value has been divided by the denominator shown, e.g. the values for As have been divided by 100.
△ (As/100) ■ (Se/1000) ▲ (Fe/10)
○ (Cu/10) □ (Zn/100)

Therefore blood copper seems to have little or no effect on blood arsenic.

Selenium seems to have a relatively large antagonistic effect on blood arsenic; as the clinical stages progressed from zero to stage 2, the blood arsenic increased significantly from 0.08 to 0.16 μmol/l and selenium from 0.71 to 0.56 μmol/l. At the third and fourth stages, the level of selenium is maintained at about 0.50 μmol/l, exerting a continual antagonistic effect which causes the blood arsenic to decrease to 0.11 μmol/l. This would explain some reports that blackfoot disease patients have lower blood arsenic values, whereas most reports emphasize arsenic as a major causative agent of the disease based on the results of drinking water analyses. This antagonistic effect is not only of academic interest and worthy of further study, but also important in the clinical treatment of blackfoot disease patients.

The blood iron contents of patients decreased as the clinical stages progressed from zero to stage 4, with values changing from 10.9 to 9.04 mmol/l. Two reasons may be offered for the decrease: 1. the antagonistic effect of arsenic toward iron, and 2. the loss of haemoglobin. The iron status of the patients is also worthy of further study from both the academic and clinical points of view.

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